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Critical analysis of the extinction coefficient of chloroplast cytochrome *f*

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Abstract

In oxygenic photosynthesis the cytochrome *bf* complex links electron transport between photosystem II and photosystem I. The largest subunit of the complex is cytochrome *f*, a 32-kDa polypeptide that is anchored in the membrane by a transmembrane alpha helix located near the carboxyl end. The three-dimensional structure of the soluble domain of cytochrome *f* isolated from turnip has been determined by X-ray crystallography to 1.96 Å resolution. The structure revealed several novel features compared to previously solved soluble *c*-type cytochrome structures, including a predominant β-strand motif, the N-terminal α-amino group of a tyrosyl residue as an orthogonal ligand, and a bound internal water chain. Here we report a novel and unprecedented extinction coefficient for cytochrome *f*. Using the pyridine hemochrome assay, the reduced minus oxidized extinction coefficient for the soluble domain of turnip cytochrome *f* was 26 ± 1 mM^{-1} cm^{-1} for the α-band wavelength peak at 554 nm relative to the isosbestic wavelengths (534, 543.5 and 560.5 nm), and 25 ± 1 mM^{-1} cm^{-1} for spinach cytochrome *f* relative to the isosbestic wavelengths (533.5, 543.3 and 560.2). The extinction coefficients reported here are significantly higher than previously published values for cytochrome *f*. We believe earlier determinations underestimated the cytochrome *f* extinction coefficient and that the same is likely true for commonly used extinction coefficients of cytochrome b_6. The cytochrome *f* extinction coefficient is large compared to most other c-type cytochromes, which could be due to the unique axial ligand of the cytochrome *f* heme. Polarographic measurements show the midpoint potential of soluble turnip cytochrome *f* to be 362 ± 5 mV at pH 7.5. The midpoint potential was pH-independent from 5.0 to 8.5, and pH-dependent from pH 8.5 to 10.5 (-58 mV/pH unit) with a pK on the oxidized form near 9. Storage of some samples of purified turnip and spinach cytochrome *f* at -20°C modified the heme environment in a fraction of the protein, shifting the midpoint potential to near -165 mV (pH 7.5) and the peak of the α-band absorption spectrum from 554 nm to 552 nm.

Keywords: Cytochrome bf; Heme protein; Midpoint potential; Pyridine-hemochrome assay

Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; E_m, midpoint oxidation–reduction potential; FeCN, potassium ferricyanide; MES, 2-(*N*-morpholino)ethanesulfonic acid; S.D., standard deviation; Tris, tris(hydroxymethyl)aminomethane.

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1. Introduction

The cytochrome *bf* complex is an integral component of the photosynthetic membranes of all oxygenic organisms (reviewed in [1,2]). The complex transfers electrons from photosystem II to photosystem I by catalyzing the oxidation of plastoquinol and the reduction of plastocyanin or cytochrome c_6. The redox reactions are coupled to proton translocation across the photosynthetic membrane, which contributes to the proton electrochemical gradient required for ATP synthesis. The complex is composed of four large subunits and at least three small hydrophobic subunits, and contains one FeS center, two *b*-hemes, and a *c*-type heme associated with cytochrome *f*. The terminal electron acceptor in the complex is the 32-kDa cytochrome *f* polypeptide. Cytochrome *f* is anchored to the thylakoid membrane by an α-helix located near the carboxyl end of the protein. The remainder of the protein, including the heme-binding domain, is exposed to the inner aqueous phase of the thylakoid vesicle [3]. Although the structure of the cytochrome *bf* complex is unknown, recent X-ray analysis has revealed the three-dimensional structure of the soluble domain of turnip cytochrome *f* (residues 1–250, $M_r = 27.5$ kDa) to 1.96 Å resolution [3,4]. Comparison of the structure of cytochrome *f* with other *c*-type cytochromes reveals some unprecedented differences. These include the unusual axial heme ligation of cytochrome *f*, provided by the α-amino-group of the N-terminal tyrosine. In other *c*-type cytochromes axial heme ligation is typically provided by methionine or histidine (or lysine under alkaline conditions) [3,5]. In addition, the secondary structure in contrast to soluble cytochrome c is predominantly β-strand, and cytochrome *f* contains a unique buried linear five water chain that is H-bonded to the histidine heme ligand.

Here we report the extinction coefficients of cytochrome *f* purified from turnip and spinach and the pH dependence of the midpoint potential of turnip cytochrome *f*. Extinction coefficients were determined using the pyridine hemochrome assay as described by Berry and Trumpower [6] to quantitate the amount of heme. Using this technique, it was found that the extinction coefficient in the α-band of reduced cytochrome *f* at its spectral peak is about 30% higher than several previously reported values for cytochrome *f* [7–9]. The experimental approach was tested by measuring the extinction coefficient of horse heart cytochrome *c* which was found to agree with previously published values.

2. Materials and methods

2.1. Purified cytochromes

The soluble 252 residue domain of cytochrome *f* was isolated from turnip (*Brassica campestris*) as described elsewhere [3], or was purchased from Sigma (St. Louis, MO). Spinach (*Spinacia oleracea*) cytochrome *f* (consisting of the complete polypeptide) and horse heart cytochrome c were obtained from Sigma (St. Louis, MO). All cytochrome *f* samples appeared as a single band on SDS-PAGE stained with Coomassie Blue.

2.2. Absorption spectroscopy

Most spectroscopic measurements used a DW2 SLM/Aminco Spectrophotometer (Rochester, NY) modified by On-Line Instrument Systems, Inc. (Bogart, GA) at a half-bandwidth of 0.3 nm. Some measurements were done using a Hewlett-Packard HP8452A Diode-Array Spectrophotometer at a half-bandwidth of 2 nm. Care was taken to ensure that the chemical titrants did not contribute to the cytochrome absorption spectra. Measurements were done at 22–25°C using a one cm pathlength cuvette.

2.3. Heme quantitation

The heme concentration of our samples was determined using the pyridine hemochrome assay as described by Berry and Trumpower [6]. The technique depends on replacing the axial ligands of the covalently bound heme in cytochrome *f* by pyridine, thereby creating the pyridine hemochrome for which the extinction coefficient is known. The conversion of the cytochrome heme to the pyridine hemochrome was carried out in a cuvette containing 1 ml total reaction volume. First, purified cytochrome *f* (50 μl of stock solution containing 20–60 μM cytochrome) was suspended in 518 μl H_2O, to which 32 μl ferricyanide (10 mM stock solution) was added to oxidize the cytochrome. Then 300 μl of pyridine

(30% by volume) was added, followed by 100 μl of 1 N NaOH (0.1 N final concentration). After thorough mixing the cuvette was placed in the dark for 5 to 30 min. (The duration of the incubation period did not alter the results.) Next, the oxidized pyridine hemochrome spectrum was recorded from 520 to 580 nm. To ensure that the sample was fully oxidized, additional ferricyanide was added (final concentration 640 μM) and a second oxidized spectrum recorded. To reduce pyridine hemochrome a few grains of solid sodium dithionite were added to the sample, after which the reduced spectrum was recorded. Additional dithionite was added to ensure complete reduction. Varying the NaOH concentration (final concentration 0.25 N), the cytochrome concentration, or total reaction volume, did not change the results. The reduced minus oxidized spectrum of pyridine hemochrome was obtained by subtraction of the oxidized from the reduced spectrum. Potassium ferricyanide, sodium dithionite, ascorbic acid, and hydroquinone were purchased from Sigma (St. Louis, MO).

2.4. Cytochrome absorption spectra

Cytochrome spectra were determined as described above for the hemochrome pyridine assay except that pyridine and NaOH were omitted, and the cytochromes were suspended in buffer containing 50 or 100 mM NaH_2PO_4 (pH 7.5). The cuvette contained 50 μl cytochrome (from stock solution of 20–60 μM), 918 μl buffer, and 32 μl FeCN (10 mM or 100 mM stock solution). After the oxidized spectrum of the cytochrome was recorded, the sample was reduced by μl additions of sodium dithionite (200 μM to 2 mM final concentration) or by the addition of a few crystals of dithionite, or in some cases by addition of ascorbate or hydroquinone from stock solutions. Dithionite was suspended in 0.1 M Tris, pH 9.0, and bubbled with argon to stabilize the stock solution. In some potentiometric experiments cytochrome spectra were measured under anaerobic conditions. To achieve anaerobicity the buffer containing the cytochrome was bubbled with high purity argon for 10 min. The concentration of oxygen was decreased further by adding glucose oxidase (Sigma) (50 units/ml), glucose (1 or 10 mM) and catalase (Sigma) (1000 units/ml) (Sigma) as described elsewhere [10].

2.5. Extinction coefficients

Cytochrome extinction coefficients were determined by comparison of the reduced minus oxidized absorption spectrum of the cytochrome with that of the pyridine hemochrome as described elsewhere [6]. The concentration of the pyridine hemochrome was determined using the extinction coefficient given by Berry and Trumpower [6] for the reduced–oxidized spectrum (24 mM^{-1} cm^{-1} at 550–535 nm).

2.6. Potentiometric redox titrations

Potentiometric redox titrations were done using a Radiometer P101 platinum electrode in combination with a Radiometer K401 calomel reference electrode as described by Dutton [11]. The ambient redox potential was calibrated by a saturated solution of quinhydrone [12]. The redox state of cytochrome *f* was determined by its α-band reduced minus oxidized absorption spectrum. Absorbance measurements were done using a DW2 SLM/Aminco Spectrophotometer modified by OLIS (Bogart, GA). Cytochrome *f* was suspended in 20 mM KCl and 100 mM NaH_2PO_4 at pH values from 6.5 to 7.5. MES (100 mM), Tris-HCl (10 mM) or CAPS (100 mM) were used as buffers at pH values 5, 8.5–10.5, and 10.5, respectively. Potassium ferricyanide was used as an oxidant and ascorbate or sodium dithionite were used as reductants. A redox buffer 'cocktail' consisting of 2-anthraquinonesulfonate ($E_{m7} = -225$ mV), hydroquinone ($E_{m7} = +280$ mV), duroquinone ($E_{m7} = +68$ mV), 2-hydroxy-1,4-naphthoquinone ($E_{m7} = -139$ mV), 1,4-naphthoquinone ($E_{m7} = +60$ mV) and 1,2-naphthoquinone ($E_{m7} = +135$ mV) each at 20 μM) was used to mediate between the membrane and the electrode [11,12]. Titrations done in the reductive or oxidative direction gave identical results. Midpoint potentials were determined from the best fit to the Nernst equation using Kaleidagraph (v. 3.0.5) (Synergy Software, Reading, PA).

3. Results

3.1. Extinction coefficients

The extinction coefficient of the soluble domain of turnip cytochrome *f* (residues 1–250) was deter-

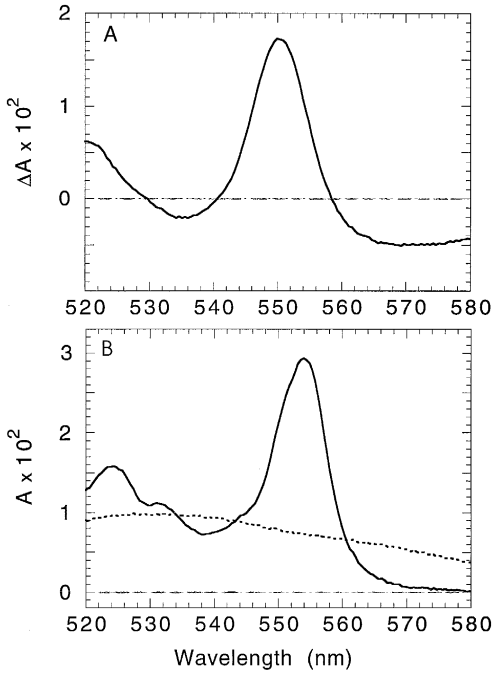

Fig. 1. (A) Reduced minus oxidized absorption spectrum of the pyridine hemochrome produced using turnip cytochrome *f*. The pyridine hemochrome was created by suspending turnip cytochrome *f* in medium containing 30% pyridine and 0.1 N NaOH as described in the text. Ferricyanide (320 μM) was used as the oxidant and dithionite (added as crystals) as the reductant. (B) Absolute reduced and oxidized α-band absorption spectra of turnip cytochrome *f* suspended in a buffer containing 100 mM NaH_2PO_4 at pH 7.5. The concentration of heme was identical in (A) and (B) (final concentration 0.8 μM).

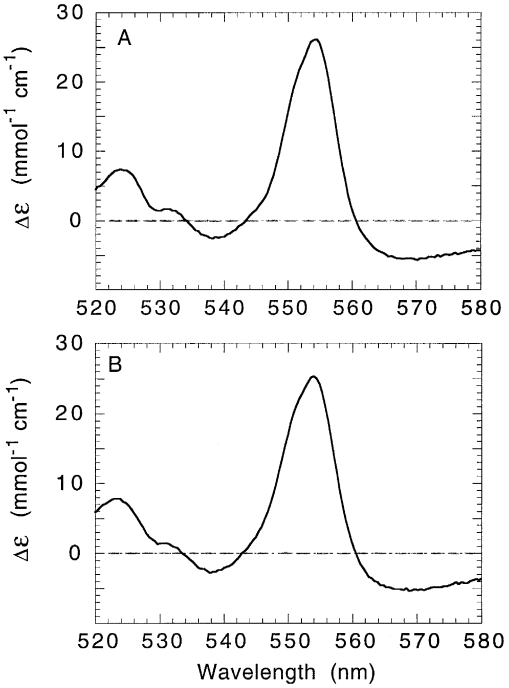

Fig. 2. (A) Reduced minus oxidized extinction coefficient of turnip cytochrome *f*. (B) Reduced minus oxidized extinction coefficient of spinach cytochrome *f*. The procedure for calculating the extinction coefficient is described in the text.

mined by converting the cytochrome heme to the pyridine hemochrome as described above. Using the Beer-Lambert law and the millimolar extinction coefficient of the pyridine hemochrome, the concentration of heme in the sample was calculated from the reduced minus oxidized absorption spectrum of the pyridine hemochrome (Fig. 1A). The absolute reduced and absolute oxidized spectra of turnip cytochrome *f* (Fig. 1B) were recorded in the α-band region at the same heme concentration as in Fig. 1A. These measurements are sufficient to calculate the reduced minus oxidized millimolar extinction coefficient of turnip cytochrome *f* from 520 to 580 nm (Fig. 2A). The extinction coefficient for the reduced

Table 1
Comparison of cytochrome *f* extinction coefficients determined using the pyridine hemochrome assay (reduced (ϵ) and reduced minus oxidized ($\Delta\epsilon$))

Cytochrome *f* (source)	ϵ (554–580 nm) (mM^{-1} cm^{-1})	$\Delta\epsilon$ (554 nm) [a] (mM^{-1} cm^{-1})	$\Delta\epsilon$ (554–540 nm) (mM^{-1} cm^{-1})	Reference
Turnip	38	26 [b]	29 [b]	(this work)
Turnip	–	26 [c]	28 [c]	Matsuzaki et al. [15]
Spinach	36	25 [b]	28 [b]	(this work)

[a] Measured from the α-band peak to the isosbestic wavelengths.
[b] S.D. $\pm$ 1, $n = 9$ for turnip and $n = 4$ for spinach.
[c] Recalculated from the original work using the reduced minus oxidized extinction coefficient for c-type pyridine hemochrome of 24 mM^{-1} cm^{-1} as given by Berry and Trumpower [6]. See text for further details.

form of turnip cytochrome f is 37.9 mM^{-1} cm^{-1} for the wavelength pairs 554–580 nm (Table 1). The reduced minus oxidized extinction coefficient for turnip cytochrome *f* is 26.2 ± 1 mM^{-1} cm^{-1} for the α-band peak at 554 nm relative to the isosbestic wavelengths, 28.5 mM^{-1} cm^{-1} for the wavelength pair 554–540 nm, and 30.0 mM^{-1} cm^{-1} at 554 nm relative to a base line drawn between the troughs at 538 and 568 nm. The peak of the cytochrome *f* reduced minus oxidized spectrum in the α-band is 554.0 ± 0.4 nm (ave. of 8 measurements) and the half-bandwidth of the spectrum is 8.6 ± 0.4 nm (ave. of 3 measurements) (calculated from the minimum at 538.5 nm to the peak). The isosbestic points for the reduced minus oxidized spectrum are 534, 543.5, and 560.5 nm. The cytochrome *f* extinction coefficient is constant over the pH range from 5.0 to 9.5.

The same procedure was followed for calculating the extinction coefficient of spinach cytochrome *f* (Table 1). The extinction coefficient for the reduced form of spinach cytochrome f is 36.5 mM^{-1} cm^{-1} for the wavelength pairs 554–580 nm. The reduced minus oxidized extinction coefficient for spinach cytochrome *f* is 25.2 ± 1 mM^{-1} cm^{-1} for the α-band peak at 554 nm relative to the isosbestic wavelengths, 27.8 mM^{-1} cm^{-1} for the wavelength pair 554–540 nm, and 28.7 mM^{-1} cm^{-1} at 554 nm relative to a base line drawn between the troughs at 538 and 568 nm (Fig. 2B). The isosbestic points are 533.5, 543.3 and 560.2 nm.

To test the pyridine hemochrome assay we measured the extinction coefficient of equine cytochrome *c*. These results gave an extinction coefficient of 21.0 ± 0.8 mM^{-1} cm^{-1} at 550 nm, measured from the isosbestic wavelength at 542.5 nm (data not shown). This value is in close agreement with extinction coefficients for equine cytochrome *c* given by van Gelder and Slater [13] (20.3 mM^{-1} cm^{-1} and 21.1 mM^{-1} cm^{-1}) and Massey [14] (21.0 mM^{-1} cm^{-1}). It is noteworthy that these determinations [13,14] did not depend on the pyridine hemochrome assay.

For accurate determination of cytochrome extinction coefficients it is essential that all of the cytochrome heme be converted to the pyridine hemochrome in the assay. Two features of the pyridine hemochrome spectrum shown in Fig. 1A indicate that the conversion of cytochrome *f* is complete. First, the peak of the absorption spectrum is shifted from 554 nm, the α-band absorption peak for cytochrome f, to 550 nm, the α-band absorption peak for the pyridine hemochrome [6]. Second, the shape of the spectrum is identical to that of the pyridine hemochrome produced using equine heart cytochrome *c* (data not shown) and to the spectrum for cytochrome c pyridine hemochrome shown by Berry and Trumpower [6].

3.2. Midpoint potentials

The equilibrium redox behavior of turnip cytochrome *f* is shown in Fig. 3A. The midpoint potential at pH 7.5 is 362 mV $\pm$ 5 mV, which is

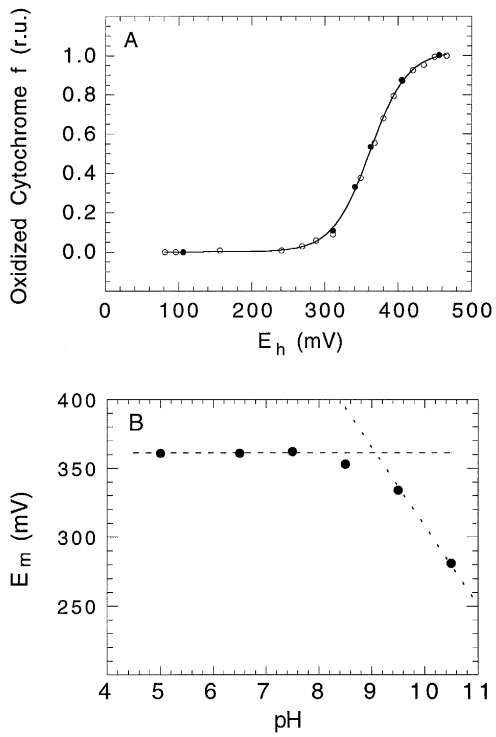

Fig. 3. (A) Potentiometric titration of turnip cytochrome *f*. The cytochrome was suspended in medium containing 100 mM NaH_2PO_4 (pH 7.5) and 20 mM KCl. Experimental details are given in the text. The titration was the same in the reductive (open circles) and oxidative direction (closed circles). The smooth curve was generated by the Nernst equation giving the best fit to the data using the program Kaleidagraph. The midpoint potential and the number of electrons were selected as free parameters. The calculated midpoint potential is 362 ± 5 mV and the number of electrons is 1.00 ± 0.02. (B) The pH dependence of the redox midpoint potential of turnip cytochrome *f*.

similar to values found for cytochrome *f* from other species (reviewed in [2]) and to that recently determined by Martinez et al. [4]. In the pH range from 5.0 to 8.0 the midpoint potential is constant, and above pH 8.5 the midpoint potential is pH-dependent (-58 mV/pH unit) (Fig. 3B). The pH dependence of the redox behavior of turnip cytochrome *f* is similar to that of parsley cytochrome *f* determined by Davenport and Hill [15] and to that shown by Martinez et al. for turnip [3].

3.3. Modification of cytochrome f by freezing

In some cases freezing cytochrome *f* in buffered solutions (50–100 mM NaH_2PO_4, pH 7.5) at -20°C for periods of a month or more modified the absorption spectrum and redox behavior of cytochrome *f* isolated from turnip or spinach. The effect was most easily seen by chemical difference spectra. An ascorbate minus ferricyanide spectrum gave an absorbance peak at 554 nm, as is seen for native cytochrome f, but only 65–75% of the cytochrome was reduced. A redox titration of the ascorbate reducible 554 nm peak gave a midpoint potential near 360 mV, as for native spinach cytochrome *f*. However, addition of dithionite produced an additional absorbance increase, with a peak at 552 nm (data not shown). A redox titration of this peak revealed a midpoint potential of -160 ± 10 mV at pH 7.5 (Fig. 4). Turnip cytochrome f also revealed a low potential form ($E_{m,7.5} = -160 \pm 10$ mV (average of three samples)). For dilute spinach or turnip cytochrome *f* samples (20–60 μM) frozen at -20°C for a month or more the low potential peak typically represented 25–30% of the total α-band absorbance change.

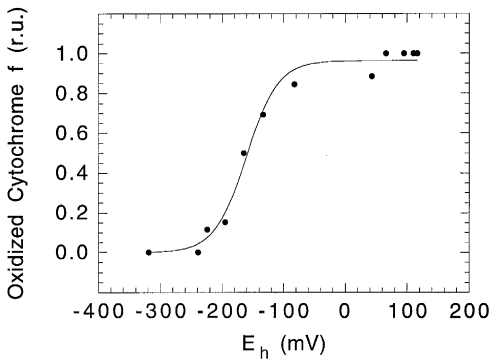

Fig. 4. Potentiometric titration of the low potential component of spinach cytochrome *f* observed in some samples that had been stored at -20°C in 100 mM NaH_2PO_4 buffer (pH 7.5) at a cytochrome concentration of approx. 60 μM. For the redox titration cytochrome *f* was suspended in 100 mM NaH_2PO_4 buffer (pH 7.5). The smooth curve was drawn from the Nernst equation giving the best fit to the data using the program Kaleidagraph. The midpoint potential and the number of electrons were selected as free parameters. The calculated midpoint potential of the low-potential component is 160 ± 5 mV and the number of electrons is 0.96 ± 0.04.

4. Discussion

4.1. Extinction coefficients of turnip and spinach cytochrome f

The reduced minus oxidized extinction coefficients in the α-band regions of cytochrome *f* from turnip and spinach are shown in Fig. 2A and Fig. 2B, respectively. It is noteworthy that the extinction coefficient of cytochrome *f* is larger than that of most soluble *c*-type cytochromes [16]. The large extinction coefficient may be due to the unique α-amino group of the N-terminal tyrosine that serves as one of the axial ligands of cytochrome *f*. It is known that the intensity of the Q_o (or α-) band is sensitive to structural perturbations [17]. In this context it should be noted that the high resolution structure of turnip cytochrome *f* does not indicate any obvious bending of the ligand bond between the N-terminal amino group and the heme iron (S.E. Martinez, personal communication). In mitochondria and some bacteria the cytochrome bc_1 complex serves a similar role to that of the cytochrome *bf* complex and has similar redox centers. However, cytochrome c_1, which serves the same role as cytochrome *f*, does not appear to have an α-amino group axial ligand [18] and has a lower extinction coefficient with published values ranging from 17.5 to 20.1 mM^{-1} cm^{-1} [19,20].

4.2. Comparison of cytochrome f extinction coefficients with previously published values

Previous measurements of the extinction coefficient of cytochrome *f* are significantly lower than the values determined here [7–9]. Forti et al. [7] obtained a value of 22 mM^{-1} cm^{-1} (554–540 nm) for the reduced minus oxidized extinction coefficient of parsley cytochrome *f* and Davenport [8] obtained a value

of 19 mM^{-1} cm^{-1} (554–540 nm) for spinach cytochrome *f*. However, both of these determinations calculated the extinction coefficient based on the total iron content of the sample rather than heme content. Any non-heme iron in the sample would cause an underestimation of the extinction coefficient, which in our view makes these determinations less reliable than those that measure the heme content.

The only published cytochrome *f* extinction coefficients we are aware of that determined the heme content are those of Matsuzaki et al. [21] and Nelson and Neumann [9], who both used the pyridine hemochrome assay. Matsuzaki et al. [21] obtained a value for turnip cytochrome *f* for the reduced minus oxidized extinction coefficient of 24 mM^{-1} cm^{-1} at 554 nm relative to the isosbestic points based on a pyridine hemochrome reduced minus oxidized extinction coefficient of 22.3 mM^{-1} cm^{-1} (550–535 nm). Nelson and Neumann [9] determined a reduced minus oxidized extinction coefficient of 20.3 mM^{-1} cm^{-1} for lettuce cytochrome *f* in cytochrome *bf* complexes at 554 nm relative the isosbestic points based on a pyridine hemochrome reduced minus oxidized extinction coefficient of 21 mM^{-1} cm^{-1} (550–535 nm). These values for the cytochrome *f* extinction coefficient, including ours, depend on the accuracy of the extinction coefficient of the pyridine hemochrome. We used the value recommended by Berry and Trumpower [6], who critically analyzed and tested previously published values. In view of the arguments put forth by Berry and Trumpower, based largely on consensus values found in the literature and their own measurements, we think an extinction coefficient of 24 mM^{-1} cm^{-1} is the most reliable.

If we recalculate the extinction coefficient of Matsuzaki et al. [21], using 24 mM^{-1} cm^{-1} for the pyridine hemochrome, the reduced minus oxidized value is 26 mM^{-1} cm^{-1} (α-band peak relative to the isosbestic points (Table 1)), which is in agreement with our data presented for turnip and spinach (Table 1). Recalculating the extinction coefficient of Nelson and Neumann [9] gives 23 mM^{-1} cm^{-1}.

4.3. Consequences of a revised extinction coefficient for quantitation of cytochrome f

There is strong evidence that the stoichiometric ratio of cytochrome *f* to cytochrome b_6 in the cytochrome *bf* complex is 1:2 (reviewed in [2]), which is supported by X-ray analysis of the cytochrome bc_1 complex from beef heart mitochondria (C.A. Yu, personal communication). The fact that many spectroscopic studies using a cytochrome *f* differential extinction coefficient near 20 mM^{-1} cm^{-1} [22] confirm this stoichiometry (e.g., [23–25]) would at first glance cast doubt on the higher extinction coefficients presented here. However, these spectroscopic determinations depend on quantitation of cytochrome b_6. Published values for the reduced minus oxidized extinction coefficient of cytochrome b_6 exhibit a wide range, from 17 mM^{-1} cm^{-1} [26] to 24 mM^{-1} cm^{-1} [9] (for the wavelength pairs 563–575 nm). Examination of published spectra for cytochrome *bf* complex [24,25] our data on the cytochrome *f* extinction coefficient indicate that to maintain a stoichiometry of 1:2 in the cytochrome *bf* complex, the extinction coefficient of cytochrome b_6 would need to be nearly as large as the extinction coefficient of cytochrome *f*. Typically a small value for cytochrome b_6 in the range of 17–20 mM^{-1} cm^{-1} has been chosen (e.g., [24,25]), which matched the low value for cytochrome *f* (20 mM^{-1} cm^{-1} for the wavelength pair 554–540 nm), thereby giving a stoichiometry of 1:2. If the larger value of 24 mM^{-1} cm^{-1} for the extinction coefficient of cytochrome b_6 is used [9] together with the values given in Table 1, the calculated ratio of cytochrome *f* to cytochrome b_6 would near 1:2. It is noteworthy that cytochrome *b* in the mitochondrial cytochrome bc_1 complex, which is analogous to cytochrome b_6 in the cytochrome *bf* complex, has a large extinction coefficient with values ranging from 25.6 mM^{-1} cm^{-1} to 28.0 mM^{-1} cm^{-1} (determined by the pyridine hemochrome assay) [20,27]. Our data for cytochrome f lead us to suggest that cytochrome b_6, like mitochondrial cytochrome *b*, may have a large extinction coefficient.

Several studies have determined the amount of cytochrome *f* in the photosynthetic membrane, usually on a chlorophyll basis (e.g., [28,29]). These determinations typically used an extinction coefficient for cytochrome *f* near 20 mM^{-1} cm^{-1} (554–540 nm). Using a larger extinction coefficient (Table 1) would lower the cytochrome *f* content by 30%. In most of these studies the conclusions depend on the relative change in the concentration of cytochrome *f* versus a growth parameter, e.g., light intensity [29],

therefore a systematic error in the absolute concentration cytochrome *f* would not alter the conclusions. One exception is the work of Graan and Ort [30] in which they determined the number of DBMIB binding sites on the cytochrome *bf* complex. Two lines of evidence, one based on the concentration of cytochrome *f* and the other on the concentration of the Rieske FeS center, led them to conclude the cytochrome bf complex operates as a dimer. Reanalysis of the stoichiometries based on a revised cytochrome *f* extinction coefficient (Table 1) would make the ratio of DBMIB binding sites to cytochrome *bf* equal 0.84, a ratio that does not support the operational dimer interpretation, at least for the plastoquinol oxidation site.

4.4. Midpoint potential of turnip cytochrome f

The cytochrome *f* heme is located in the aqueous lumenal phase of the photosynthetic membrane. The pH of the lumen is controlled largely by the light intensity and typically varies between pH 5.5 and 7.5 (e.g., [31]). Over this pH range the equilibrium midpoint potential of turnip cytochrome *f* is constant (approx. 360 mV) (Fig. 4). Above pH 8.5 the midpoint potential is pH-dependent, decreasing 58 mV for every pH unit increase. Fig. 3B indicates a pK on the oxidized form of cytochrome *f* at 9. Based on the structure of cytochrome *f*, Martinez et al. [4] considered several amino acid residues, as well as heme propionates that could account for the observed pK, and inferred that deprotonation of the histidine imidazole at position 25 may be responsible.

4.5. Cytochrome f heterogeneity induced by freezing

In some cases, storing samples of isolated cytochrome *f* from turnip or spinach at -20°C created a low potential form of the heme ($E_{m,7.5} = -160$ mV). The low midpoint potential of the cytochrome *f* heme is similar to that for iron protoporphyrin IX ($E_{m,7.5} = -145$ mV [12]). The redox heterogeneity observed for cytochrome *f* may be similar to that observed for cytochrome c_1 samples, in which it was observed that after ageing a fraction of the cytochrome was not reduced in the presence of ascorbate [32]. Cytochrome c_1 of the cytochrome bc_1 complex plays a role analogous to that of cytochrome *f*. We did not observe this freezing-induced heterogeneity in horse heart cytochrome *c* or cytochrome c_6 from *Synechocystis* 6803. The effect may be due to the unique N-terminus amine that serves as sixth ligand for the heme of cytochrome *f* [3,4], although this explanation would not account for the ageing-induced modification of cytochrome c_1, which does not have an amine ligand. The possibility was considered that oxygen may replace the amine ligand in the modified samples. However, it was found the low potential spectrum was present in cytochrome *f* samples under anaerobic conditions, making it unlikely that oxygen plays a role in this phenomenon.

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References

[1] Cramer, W.A., Soriano, G.M., Ponomarev, M., Huang, D., Zhang, H., Martinez, S.E. and Smith, J.L. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 477–508.
[2] Kallas, T. (1995) in Molecular Biology of Cyanobacteria (Bryant, D.A., ed.). pp. 259–317, Kluwer Press.
[3] Martinez, S.E., Huang, D., Szczepaniak, A., Cramer, W.A. and Smith, J. (1994) Structure 2, 95–105.
[4] Martinez, S.E., Huang, D., Ponomarev, M., Cramer, W.A. and Smith, J. (1996) Protein Sci. 5, 1081–1092.
[5] Meyer, T.E. and Kamen, M.D. (1982) Adv. Protein Chem. 35, 105–212
[6] Berry, E.A. and Trumpower, B.L. (1986) Analyt. Biochem. 161, 1–15.
[7] Forti, G., Bertole, M.L., Zanetti, G. (1965) Biochim. Biophys. Acta 109, 33–40.
[8] Davenport, H.E. (1971) in Proc. IInd Intern. Congress on Photosyn. Res. (Forti, G., Avron, M. and Melandri, A., eds.) 2, pp. 1593–1601, The Hague: Junk.
[9] Nelson, N., and Neumann, J. (1972) J. Biol. Chem. 247, 1817–1824.
[10] Nedbal, L., Samson, G., and Whitmarsh, J. (1992) Proc. Natl. Acad. Sci. USA 89, 7929–7933.
[11] Dutton, P.L. (1978) Meth. Enzymol. 54, 411–435.
[12] Clark, W.M. (1960) Oxidation–Reduction Potentials of Organic Systems, Waverly Press, Baltimore, MD.
[13] Van Gelder, B.F. and Slater, E.C. (1962) Biochim. Biophys. Acta 58, 593–595.
[14] Massey, V. (1959) Biochim. Biophys. Acta 34, 255–256.

[15] Davenport, H.E. and Hill, R. (1952) Proc. R. Soc. London, Ser. B 139, 327–345.
[16] Meyer, T. E. and Kamen, M. D. (1982) Adv. Protein Chem. 35, 105–212.
[17] Makinen, M.W. and Chung, A.K. (1983) In: Iron Porphyrins I, eds. A.B.P. Lever and H.B. Gray, p. 167. Addison-Wesley, Reading, MA.
[18] Finnegan, M.G., Knaff, D.B., Qin, H., Gray, K.A., Daldal, F., Yu, L., Yu, C.-A., Kleis-San Francisco, S. and Johnson, M.K. (1996) Biochim. Biophys. Acta 1274, 9–20.
[19] Yu, C.A., Yu, L. and King, T.E. (1972) J. Biol. Chem. 247, 1012–1019.
[20] Berden, J.A. and Slater, E.C. (1970) Biochim. Biophys. Acta 216, 237–249.
[21] Matsuzaki, E., Kamimura, Y., Yamasaki, T. and Yakushiji, E. (1975) Plant Cell Physiol. 16, 237–246.
[22] Cramer, W.A. and Whitmarsh, J. (1977) Ann. Rev. Plant Physiol. 28, 133–172.
[23] Bendall, D. and Rolfe, S. (1987) Meth. Enzymol. 148, 259–273.
[24] Huang, D., Everly, R.M., Cheng, R.H., Heymann, J.B., Schägger, H., Sled, V., Ohnishi, T., Baker, T.S. and Cramer, W.A. (1994) Biochemistry 33, 4401–4409.
[25] Pierre, Y., Breyton, C., Kramer, D. and Popot, J.-L. (1995) J. Biol. Chem. 270, 29342–29349.
[26] Stuart, A.L. and Wasserman, A.R. (1973) Biochim. Biophys. Acta 314, 284–297.
[27] Zaugg, W.S. and Rieske, J.S. (1962) Biochem. Biophys. Res. Comm. 9, 213–217.
[28] Whitmarsh, J. and Ort, D. (1984) Arch. Biochem. Biophys. 231, 378–389.
[29] Lee, W.J. and Whitmarsh, J. (1989) Plant Physiol. 89, 932–940.
[30] Graan, T. and Ort, D. (1986) Arch. Biochem. Biophys. 248, 445–451.
[31] Nishio, J. and Whitmarsh, J. (1993) Plant Physiol. 101, 89–96.
[32] Yu, L., Dong, J.-H. and Yu, C.-A. (1986) Biochim. Biophys. Acta 852, 203–211.